Protocol by Virginia Rich, vrich@mit.edu, graduate student in the DeLong Lab
Last modified 2/12/08

96-well DNA Extraction Protocol from 25mm 0.2um filters

As used in Rich, Konstantinidis, and DeLong. 2008.

**** This protocol is NOT suitable for cleaning up already-extracted DNAs! They do not bind effectively to the DNEasy Tissue Columns using this protocol, as written.****

Protocols relevant to the development of this one:

- the lab's latest "**DNA/RNA Extraction from Large Volume Steripak Filters**" protocol, used after the recent Hawaii cruise to extract community DNA for library-making.

- the extraction protocol described in **Suzuki et al 2001**, the upwelling plume paper; they extracted DNA from 13mm Supor-200 filters used to filter 30ml of seawater. They froze filters in 180um of a homemade lysis buffer: 20mM Tris, pH 8; 2mM EDTA, pH8, 1.2% TritonX, and 20mg/ml lysozyme. Once thawed at a later time, they incubated 1hr at 37ºC. Added 1ul of 15 Kunitz U/ml RNaseA, incubated 5" @ RT. Then added 25ul of a 25mg/ml ProK (final conc. 3.05mg/ml). mixed by vortexing, and then followed **Qiagen DNeasy Tissue kit, protocol for Gram-positive Bacteria**: incubate 1-3hrs at 55ºC, vortex 15sec, add 200ul BufferAL (this contains guanidine salt and Tween), incubate 70ºC 10", add 200ul 96-100% EtOH, mix thoroughly by vortexing, and apply to columns.

- **Boström *et al.* 2004** (Ake Hagström's lab) L&O Methods paper, vol. 2:365-373. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Their lysis buffer was 400mM NaCl, 750mM sucrose, 20mM EDTA, 50mM Tris pH 9, and 1mg/ml final conc. lysozyme. Incubated 30" at 37ºC (they also tried 120" but saw worse extraction efficiency, prob due to nuclease activity Tracy thought). Then added SDS to final conc. of 1%, and ProK to final conc. of 100ug/ml. They incubated at 55ºC, and saw that an overnight treatment was far better than a shorter treatment. They then proceed with a phenol extraction protocol. Didn't end up using their protocol but mostly because the Steripak one wasn't too diff, and although it only has a 2hr ProK incubation, it also has a 6.5x higher ProK conc.

- converstations with Tracy. He typically used 1mg.ml final lysozyme conc., 200ug/ml RNase

- **DNeasy 96 Tissue Handbook** protocol

- **ExcelaPure 96-well UF PCR Purification** protocol

Expected DNA yield: rule of thumb is about 1fg per genome, 10^6 genomes per ml, so 1ng DNA per ml, so 1ug DNA per L. SO, I filtered 250mls – 1L, so I should expect (best case scenario) 250ng – 1ug DNA out.

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Materials

2 full ice buckets
0.2um syringe-filters (e.g. Pall Corp. # 4192, Acrodisc 25mm syringe filters, sterile)
syringes, size depends on volume buffer required; usually 5ml, 20ml and 60ml
rotating hyb oven set to 37ºC
rotating hyb oven set to 55ºC
later, a heat-block or hyb oven (depending on your lysis set-up) set to 70ºC
96-well DNA size-exclusion columns, e.g. Edge Biosystems ExcelaPure 96-well UF Plate, #4050208.
Qiagen DNeasy 96 Tissue Kit, Qiagen #69581
1M Tris, pH 8.3 (kept at my bench, but pH 8.0 – this is OK; e.g. Ambion #9855G)
0.5M EDTA (kept at my bench, e.g. Ambion #9260G)
Sucrose (e.g. stockroom; Mallinckrodt Chemicals #8360-04)
10% SDS (kept at my bench; e.g. Ambion #9822)
Lysozyme (kept in door of freezer; e.g. Sigma #L-6876)
Proteinase K (stored frozen and dry; e.g. EMD #24568-2)
100mg/ml RNase (stored at R.T., in bench drawer; e.g. Qiagen #1018048)

Lysis Buffer: (make fresh because of sucrose)

Adapted from Steripak protocol, with addition of RNase.

Required volumes: for ½ plate 20mls is plenty – split into 15ml plus 5mls; for a whole plate 35mls is good – split into 30mls plus 5mls.

Final Concentration	For 20ml	For 35ml
40mM EDTA	1.6ml of 0.5M EDTA	2.8ml of 0.5M EDTA
50mM Tris (pH 8.3)	1.0ml of 1M Tris (pH 8.3)	1.75ml of 1M Tris
0.73M Sucrose	5.13g of Sucrose	8.98g of Sucrose

shake vigorously to dissolve
add water to appropriate final volume, shake
split into two aliquots (15+5 or 30+5); can filter-sterilize now but will be sterilizing each aliquot separately so if proceeding immediately (as you should) then no need to double-sterilize.

1. For Lysozyme & RNase Aliquot: right before use, add to one aliquot:

Final Concentration	For 15ml	For 30ml
1.15mg/ml Lysozyme	17.31mg lysozyme	34.62mg lysozyme
200ug/ml RNase 100mg/ml	30ul RNase 100mg/ml	60ul RNase

Note: Using slightly old RNase is fine – I keep mine in a drawer at R.T. and Chon and others confirmed that several-year-old RNase should be OK, no need to use extra of it.
Shake to dissolve thoroughly, then filter-sterilize through 0.2um filter.

2. For ProK Aliquot:
Weigh out minimum amount ProK, then add the appropriate amount of lysis buffer (from the second 5ml aliquot). If you used the full 50mg of ProK for 5ml of buffer that would be wasteful since you don't need it. For a half-plate you'll need about 1.5mls to have plenty, for a whole plate you'll need 3mls.

Final Concentration	For entire 5ml	For 1.5ml	For 3.0ml
10mg/ml	50mg ProK	15mg ProK	30mg ProK

shake vigorously to dissolve (it may foam a little).
filter sterilize through 0.2um syringe filter.

Protocol

Step 1: Cell Lysis & RNA removal
Source: adapted from lab Steripak protocol
- thaw filters on ice; do quick spin down
- transfer each filter to screw-top, O-ringed eppendorf tube, also on ice
Note: For my purposes my samples were very precious each time so I didn't muck around, I just used the good, strong, o-ring eppis. However, if one were doing a whole plate's worth of samples or doing this many times, then for lysis you might want to use the rack of collection microtubes in the DNeasy 96 kit, these come with tight caps and are designed for lysis and allow mutli-channel pipetting.
- add 250ul lysis buffer with RNase and lysozyme to each tube
- incubate 37ºC for 30", rotating end-over-end at angle, for optimal mixing with minimal frothing

Step 2: Protein Degradation
Source: adapted from lab Steripak protocol
- add 18.75ul of Proteinase K solution (10mg/ml made up in lysis buffer) to a final conc. of 0.65 mg/ml
- add 29.9ul 10% SDS to a final conc of 1%
- incubate at 55ºC for 2 hours, rotating end-over-end at angle
Can also let this step go overnight if needed.

Towards end of this time, turn on heat block or hyb oven to 70ºC. Put elution liquid (Buffer AE or water) into 70ºC to preheat.

Step 3: DNA Purification Through DNA-binding Columns
Principle: selective adsoption of DNA to a silica-gel-membrane.
Source: adapted from DNeasy Tissue kit protocol for Gram-positive bacteria, and from DNeasy 96 Tissue kit protocol, and from Marcelino's protocol in the upwelling paper.

Notes: According to 96 kit, vol. after steps 1&2 would be about 200ul, to which they add 410 of buffer AL/E; according to Marcelino's protocol with the old Tissue kit, after steps 1&2 vol. would be 206ul, to which they added 200ul buffer AL, incubated 10" at 70, then added 200ul EtOH. Tech support for 96 kit says to use the slightly higher ratio of buffer to lysate vol that they use in their protocol. They also confirmed that Buffer AL/E, before you add the ethanol, is the same as Buffer AL from the old kit.

Note on spins: these are very fast spins, and so balancing is important. If only doing 1 plate, Tracy suggests using a 96 deep-well plate as a balance (water squirt bottle). He says it will shake anyway but not to be alarmed. He greased the hinges of the swinging plate-holders and said they should be good til next year (2008), and that the plates do fit, barely, in our rig with room to rotate.

The max speed of our rotor is hypothetically 5650rpm but it will only accept 5250rpm; 5000rpm = "4612 x g" on the display. So, while the 96-well protocol calls for faster spins (below), we can't reach them.

Our centrifuge in the main lab does not really recognize the plate-spinning rotor; therefore, it does not correctly convert the rpm to the rcf. You can trust the rpm, but not the rcf; that you should calculate yourself.

The equation for interconverting RCF (xg) and RPM is:

RCF = (1.12 * *radius in mm*) * (RPM / 1000) ^2

Thus, our 5000rpm spins on our rotor, which has a max plate-spinning radius of 16cm but which spins the DNeasy plates at ~12cm, provides **3360 xg.**

- Add 300ul Buffer AL (=Buffer AL/E without the ethanol added)

mix thoroughly by vortexing, and spin down quickly
incubate 70ºC for 10"

- Add 300ul 96-100% EtOH

mix by vortexing vigorously, and spin down quickly

****check pH of lysate, must be <7 to get max. binding efficiency to column****

- Pipet onto 96-well spin columns, making sure not to whet the rims to avoid cross-contamination. Max lysate volume to add to spin columns at one time = 900ul. Place the 96-well column plate onto the S-block for flow-through collection; S-block supplied with kit.

seal plate with Airpore tape sheet (included in kit)
spin 5788 x g for 10", at 40ºC

Note: because we can't spin at 40ºC, I put the plate set-up into a 40ºC hyb oven while I prepare the spin balance, so it gets 2-5" at temp before being spun.

discard flow-through
place in new collection tray

(or in my case just empty out S-block thoroughly and wipe down top rims).

add additional lysate if needed and repeat spin, etc.

- Add 500ul Buffer AW1, reseal plate
spin 5788 x g 5", at 40ºC
- Add 500ul Buffer AW2, reseal plate
spin 5788 x g for 5", at 40ºC
- To dry columns, either reseal plate with new sheet and spin 5600 x g for 15" at 40ºC, atop a new collection tray, or incubate in hyb oven at 70ºC for 15"
- Transfer column plate to top of rack of "elution microbubes RS"
- Add 200ul pre-heated 70ºC Buffer AE or water, reseal plate

Note: The only reason to use Ambion water would be if you intend to hyb the crude lysate... which isn't a great idea. You really should do Step 4 for additional clean-up, and if you do Step 4 then you can get the DNA in any liquid you want at that stage. SO, you may as well elute here in Buffer AE since the elution efficiency is highest with that buffer, whose pH is 9.0. If you use water just make sure its oH is >= 7.0 to permit elution.

incubate 1" @ RT
spin 5788 x g 2" to elute

- Repeat with a second 200ul, this will increase total yield up to 25%. Or if you wanted to keep the colume small, you could use the first 200ul elution, heat it back to 70ºC, and pass it through the column again; this will increase total yield approx. 15%.
- Can freeze elutants and break here overnight, or for a while, before proceeding with Step #4, particularly if DNA is in Buffer AE which is TE, so will keep the DNA relatively stable.

<u>Step 4: Final DNA Clean-up & Concentration by Size-Exclusion Columns</u>

from the ExcelaPure 96-well UK PCR purification kit protocol

Warning: test plate & vacuum set-up before you begin

- transfer the eluted DNA to the 96-well PCR purification plate, no more than 300ul at a time!
- apply vacuum at **20 inches Hg** until dry (maybe 20-30"?) membrane will appear shiny when dry
- rinse DNA with 100ul Ambion water, apply vacuum 5-10" until dry. Rinse a second time with water if you want to make sure you've really removed all the Buffer TE.

- add 20ul dilute TE, pipette up and down 20 times and transfer to a clean 96-well plate e.g. PCR plate for temporary or permanent storage, can move to individual tubes for later ease of use.
- optional: repeat with another 20ul to ensure all retrieved. DNA stores better at higher concentrations, so elute in minimum possible volume – see expected yields, below.

Excelapure protocol calls for 100ul elution, Tracy has gone as low as 20ul

dilute TE is 1:10 regular TE.

Expected DNA yield: for a 250ml filtration, if only on the order of 10^5 cells (as in for most Hawaii samples, or for sparse MB samples) then only 25ng of DNA, so 1.25ng/ul. DNA stores better at higher conc. too.